Basolateral Efflux Transporters: A Potentially Important Pathway for the Prevention of Cholestatic Hepatotoxicity

Jonathan P. Jackson, Kimberly M. Freeman, Weslyn W. Friley,
Robert L. St. Claire III, Chris Black, and Kenneth R. Brouwer

Abstract

The hepatocyte adaptive response has been postulated to play a protective role in cholestatic disease states, including primary biliary cirrhosis where bile acid biliary efflux is reduced. Regulation of bile acid synthesis by farnesoid X receptor (FXR) has been well defined; however, bile acid transport has not. Utilizing sandwich-cultured human hepatocytes (SCHH) and B-CLEAR[®] technology, we demonstrated that basolateral efflux and not canalicular efflux (bile salt export pump [BSEP]) was the primary driver of bile acid intracellular accumulation. Following 72 hours of exposure to CDCA, decreases of total endogenous bile acid mass and CYP7A1 mRNA content were observed in SCHH consistent with FXR activation. No marked changes were observed in mRNA content of bile acid uptake transporters, however, induction of bile acid efflux transporters OST α (3.1–6.8 $\times$), OST β (21–187 $\times$), and BSEP (2.2–7.5 $\times$) mRNA content was observed. While decreases of d8-TCA biliary clearance were inconsistent with the increases in BSEP mRNA content, decreases in the intracellular concentrations of the model bile acid, d8-TCA, were observed in SCHH following CDCA exposure. Overall, these data suggest that basolateral efflux of bile acids via OST α/β is a potentially important compensatory mechanism to prevent cholestatic hepatotoxicity.

Keywords: bile acid transport, cholestatic hepatotoxicity, DILI

Introduction

THE HEPATOCYTE ADAPTIVE RESPONSE has been implicated to play a protective role in cholestatic disease states, including primary biliary cirrhosis, a disease state characterized by reduced bile acid biliary efflux.^{1,2} *In vivo*, hepatic concentrations of bile acids are tightly regulated through synthesis, metabolism, and transport mechanisms.^{1,3} These cellular processes are controlled by the master bile acid regulator, farnesoid X receptor (FXR), which acts as a bile acid sensor to carefully monitor and maintain bile acid homeostasis.^{4,5} The impact of FXR activation on bile acid synthesis has been well defined; however, its role in bile acid transport and disposition is not well understood. Studies have demonstrated that CDCA, a primary bile acid, is the most potent endogenous FXR activator.^{4–6} CDCA is rapidly conjugated with glycine and taurine, and these conjugates are bile salt export pump (BSEP) substrates.^{7,8} Other transporters are likely involved in mediating bile acid efflux in the liver, including multidrug resistance-associated proteins

(e.g., MRP3 and MRP4) and the lowly expressed heterodimer organic solute transporter (OST α /OST β).^{9–12} Studies have demonstrated that BSEP and OST α /OST β are inducible via FXR activation.^{2,13,14} However, these studies have not shown how these adaptive responses affect bile acid disposition following FXR activation.

In the present study, we utilized sandwich-cultured human hepatocytes (SCHH) from three donors and B-CLEAR technology to examine the bile acid adaptive response impact on bile acid disposition following exposure to the potent FXR agonist, CDCA.

Materials and Methods

Chemicals, cells, and reagents

Chenodeoxycholic acid, tamoxifen, DMSO, and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO). D4-glychenodeoxycholic acid, d5-taurochenodeoxycholic acid, d5-glycocholic acid, d4-cholic acid, and d5-chenodeoxycholic acid were purchased from Toronto

Qualyst Transporter Solutions, Durham, North Carolina.

© Jonathan P. Jackson et al., 2016; Published by Mary Ann Liebert, Inc. This Open Access article is distributed under the terms of the Creative Commons License (<http://creativecommons.org/licenses/by/4.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly credited.

Research Chemicals (Toronto, Canada). D8-taurocholate was purchased from Martrex, Inc. (Minnetonka, MN). D5-taurocholate (used as an internal standard) was purchased from Toronto Research Chemicals, Inc.. HPLC grade methanol and acetonitrile were purchased from World Wide Medical Products (Bristol, PA). Primary human hepatocyte cultures were seeded and maintained utilizing propriety cell culture media formulations developed at Qualyst Transporter Solutions (QTS). Cell culture base medium and supplements were purchased from Gibco (Carlsbad, CA) and Corning (Tewksbury, MA). CellTiter-Glo[®] Luminescent Cell Viability Assays were purchased from Promega (Madison, WI). All quantitative real-time polymerase chain reaction (qRT-PCR) reagents were purchased from Life Technologies (Carlsbad, CA). Pierce[™] BCA[™] Protein Assays were purchased from Thermo Fisher Scientific (Waltham, MA).

Sandwich-culture hepatocyte cultures and treatment

SCHH were prepared using QTS Transporter Certified[™] cryopreserved human hepatocytes purchased from Triangle Research Laboratories (HUM4119C and HUM4122C; RTP, NC) and Xenotech (HC3-26; Lenexa, KS). Cryopreserved hepatocytes were thawed following the manufacturer's thawing instructions. SCHH were prepared by plating cryopreserved hepatocytes suspended in the QualGro[™] Seeding Medium, QTS propriety hepatocyte seeding medium, at a density of 0.8 million viable cells/mL onto BioCoat[®] 24-well cell culture plates purchased from Corning. Following plating, cells were allowed to attach for 2–4 hours, rinsed, and fed with warm (37°C) seeding medium. Eighteen to 24 hours later, cells were fed and overlaid with QualGro Hepatocyte Culture Induction Medium supplemented with 0.35 mg/mL Matrigel[®] purchased from Corning. Cells were maintained in QualGro Hepatocyte Culture Induction Medium until consumed in studies.

SCHH were treated daily beginning on day 2 of culture for 3 days with fresh dosing solutions of d₅-CDCA or unlabeled CDCA (100 μ M) or cytotoxicity-positive control (10, 50 μ M tamoxifen) to evaluate drug transporter induction, bile acid composition, bile acid disposition, and cytotoxicity. Dosing solutions were prepared fresh daily from DMSO stock solutions and discarded after dosing of hepatocyte cultures. DMSO stock solutions were prepared at 1000 $\times$ final concentration and stored at $\leq -20^{\circ}\text{C}$. Test article stock solutions were diluted directly into the QualGro Hepatocyte Culture Induction Medium. The final organic solvent in all dosing solutions was $\leq 0.1\%$. Negative control cultures were treated with vehicle (0.1% DMSO). Twenty-four hours after last dosing, assessments of cytotoxicity, drug transporter induction, endogenous bile acid profiling, and bile acid disposition were determined.

Cytotoxicity assessment

Cell viability of hepatocytes was assessed by determining the amount of adenosine triphosphate (ATP) present after 72 hours of exposure to CDCA (100 μ M) or cytotoxicity-positive control (10, 50 μ M tamoxifen). Cellular ATP pools in SCHH were measured using CellTiter-Glo[™] Luminescent Cell Viability Assays from Promega following the manufacturer's procedures. Luminescence of samples was detected

using a BioTek Synergy4 Plate Reader. Each test condition was performed in triplicate. In addition, qualitative assessments of hepatocyte morphology were evaluated and compared to solvent controls for any morphological alteration (e.g., changes in cell shape, cytoplasmic alterations, accumulation of vacuoles suggestive of dilated organelles and lipid droplets) indicative of cytotoxicity.^{15,16}

Total RNA isolation and qRT-PCR

Following 72 hours of treatment with CDCA (100 μ M), SCHH were washed once with one volume of HBSS and lysed by addition of 0.3 mL of Qiagen RLT Buffer (Germantown, MD) supplemented with β -mercaptoethanol and frozen at -80°C . Lysates were thawed and total RNA was isolated from each treatment group, pooled from triplicate wells, using the Qiagen RNeasy kit, following the manufacturer's instructions. RNA was stored at -80°C . Isolated RNA was quantified using the Quant-iT[™] RiboGreen[®] RNA Assay Kit (Life Technologies) following the manufacturer's instructions. Pooled total RNA (500 ng) was converted to cDNA following the manufacturer's procedure for the High Capacity cDNA Archive Kit (Life Technologies). CYP7A1, small heterodimer partner (SHP) (NR0B2), fibroblast growth factor 19 (FGF19), NTCP (SLC10A1), OATP1B1 (SLCO1B1), OATP1B3 (SLCO1B3), OATP2B1 (SLCO2B1), BSEP (ABCB11), P-gp (ABCB1), MRP2 (ABCC2), MRP3 (ABCC3), MRP4 (ABCC4), BCRP (ABCG2), OST α (SLC51A), and OST β (SLC51B) cDNA from human hepatocyte cultures was analyzed from each RT reaction using gene-specific TaqMan[®] assays (Life Technologies; Table 1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was utilized as an endogenous control for gene expression analysis. Amplifications were performed on a ViiA[™] 7 Real-Time PCR System (Thermo Fisher Scientific) in relative quantification mode for 45 amplification cycles using standard conditions for TaqMan-based assays. Threshold cycle (CT) determinations were performed by the ViiA 7 system software for both target and endogenous control genes. Relative-fold mRNA content was determined for each treatment group relative to the endogenous control gene expression and the calibrator, 0.1% DMSO vehicle

TABLE 1. LIST OF TARGET GENES

<i>Target gene</i>	<i>Assay #</i>
CYP7A1	Hs00167982_m1
CYP8B1	Hs00244754_s1
SHP (NR0B2)	Hs00222677_m1
FGF19	Hs00192780_m1
NTCP (SLC10A1)	Hs00161820_m1
OATP1B1 (SLCO1B1)	Hs00272374_m1
OATP1B3 (SLCO1B3)	Hs00251986_m1
OCT1	Hs00427552_m1
BSEP (ABCB11)	Hs00184824_m1
P-GP (ABCB1)	Hs00184500_m1
MRP2 (ABCC2)	Hs00166123_m1
MRP3 (ABCC3)	Hs00978473_m1
MRP4 (ABCC4)	Hs00988717_m1
BCRP (ABCG2)	Hs01053790_m1
OST α (SLC51A)	Hs00380895_m1
OST β (SLC51B)	Hs01057182_m1

control using the ViiA 7 system software. Confidence intervals of 95% were calculated for each target gene RQ mean by the ViiA 7 system software.

Preparation of SCHH for bile acid profiling and disposition

Following 72 hours of treatment with d₅-CDCA (100 μ M) or CDCA (100 μ M), the hepatobiliary disposition of endogenous bile acids (e.g., TCA, GCA, GCDCA, and TCDCA) and d₈-TCA was determined in SCHH using the patented B-CLEAR technology (QTS, Durham, NC). Cell culture medium was removed from SCHH and stored at -80°C for endogenous bile acid composition analysis. After removal of cell culture media, the tight junctions of untreated and treated SCHH were modulated using B-CLEAR technology (QTS).^{17–20}

B-CLEAR technology

Using patented methodology (Qualyst Transporter Solutions, NC), sandwich-cultured Transporter Certified hepatocytes in parallel cultures, were washed twice with Plus (+) buffer (buffer containing Ca++) or Minus (–) buffer (buffer without Ca++). The wash solutions were removed and replaced with fresh Plus (+) or Minus (–) buffer and incubated for 10 minutes at 37°C to modulate tight junctions.^{17–20} After incubation, the Plus (+) and Minus (–) buffers were removed and replaced with a dosing solution composed of Plus (+) buffer with 2.5 μ M d₈-TCA and 4% BSA. The dosing solution was incubated at 37°C for 30 minutes. Note that a dosing solution was not utilized in measurements determining the hepatobiliary disposition of endogenous bile acids. Following the incubation, the dose solution was removed and the hepatocytes were then washed three times with ice-cold Plus (+) Buffer. The plates were frozen at -80°C until processed for bioanalysis to determine protein content, composition, and disposition of endogenous BA, and disposition of d₈-TCA or d₅-GCDCA. Protein content was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) following the manufacturer's instructions.

Bioanalysis of bile acid profiling and disposition assessment

Analytes (d₈-TCA, TCA, GCA, CA, TCDCA, GCDCA, CDCA,) were extracted from previously frozen 24-well plates containing hepatocyte lysates utilized in bile acid profiling and disposition assessments. Briefly, a volume of 500 μ L of lysis solution, 70:30 methanol:water (v:v) containing 25 nM internal standard (d₅-TCA), was added to each well. Plates were shaken for at least 15 minutes and the cell lysate solution was transferred to a Whatman® 96-well Unifilter® 25 μ m MBPP/0.45 μ m PP filter plate (7770-0062; Whatman) stacked on a 96-well deep well plate. Lysate was filtered into the deep well plate by centrifugation. The sample filtrate was evaporated, under nitrogen, to dryness and the samples were reconstituted in 120 or 150 μ L sample diluent, 60:40 methanol:water containing 4 mM ammonium acetate, and mixed for at least 10 minutes on a plate shaker. The reconstituted samples were transferred to a Millipore 0.45 μ m filter plate (MSHVN45; Millipore) and filtered into a Costar 3957 plate by centrifugation (2390 rcf for 2 minutes) and sealed with a silicone capmat before LC-MS/MS analysis. Standard and quality control samples were

prepared by spiking previously frozen analytical plates containing hepatocyte lysates with known concentrations of the stable label version of the endogenous bile acids (d₅-CDCA, d₈-TCA, d₅-TCDCA, d₄-GCDCA, d₄-CA, and d₅-GCA), using a concentration range typically spanning from 0.5 to 500 pmol/well. These lysate controls were processed as described above.

Cell culture media study samples were collected and stored frozen in a 96-well deep well block before analysis. Analytes were extracted from unknown media samples using a protein precipitation procedure. Briefly, 300 μ L of 100% methanol containing internal standard (d₅-TCA) was added to each well of a 96-well deep well plate. Media study samples (100 μ L aliquot volume) were added to the plate and the plate was allowed to shake for at least 10 minutes before transferring sample to a Whatman 96-well Unifilter 25 μ m MBPP/0.45 μ m PP filter plate (7770-0062; Whatman) stacked on a 96-well deep well plate. Samples were filtered into a clean deep well plate. The filtrate was evaporated, under nitrogen, to dryness and samples were reconstituted in 150 μ L of sample diluent, 60:40 methanol:water containing 4 mM ammonium acetate, processed, and sealed as previously described, before LC-MS/MS analysis. Separate standards and quality control samples were prepared for cell culture media study samples. Analytes were measured using standard curves of the stable label version of the endogenous bile acids (d₅-CDCA, d₈-TCA, d₅-TCDCA, d₄-GCDCA, d₄-CA, and d₅-GCA) prepared in the appropriate media. Standard and quality control media solutions were prepared by diluting spiking solutions 1:10 in appropriate media over a concentration range (typically 0.01–10 μ M). Further processing of media standard and QCs was completed as described for unknown media samples.

LC/MS/MS was performed using a Thermo Electron TSQ® Quantum Discovery MAX™ (Waltham, MA) with an Ion Max ESI source operated in negative electrospray ionization mode. Chromatographic separation for the endogenous bile acids and their deuterated counterparts (for use as analytical standards and dosing material for d₅-CDCA) was achieved using a Shimadzu binary HPLC system with LC-10ADvp pumps (Columbia, MD) equipped with a Thermo Scientific Hypersil Gold™ 100 $\times$ 1.0 mm, 3 μ m with matching guard and precolumn filter (Bellefonte, PA), using a linear gradient of 45%–100%B, at a flow rate of 0.05 mL/min, with mobile phase A as 20% methanol with 0.25 mM ammonium acetate and mobile phase B as 80% methanol with 0.25 mM ammonium acetate, both at native pH.

Data analysis

All calculations were performed using Microsoft Excel, 2010, unless otherwise stated. Statistical analysis was performed using GraphPad Prism Software (Version 6.0).

To quantify ATP depletion as a result of treatment, percent of control was calculated as the ratio of ATP content of treated samples to vehicle control (0.1% DMSO) as described in Equation (1).

Equation 1:

$$\% \text{ of Control} = \frac{\text{ATP Content}_{\text{Treatment}}}{\text{ATP Content}_{0.1\% \text{ DMSO}}} \times 100$$

Changes in mRNA content (ΔCT) were determined for each treatment group relative to endogenous control gene expression and the calibrator, 0.1% DMSO vehicle control

($\Delta\Delta CT$). The relative-fold mRNA content was determined by using the change in the threshold values [$\Delta\Delta CT$; Eq. (2)]. All relative quantitation calculations were performed using the ViiA 7 system software.

Equation (2):

$$\text{Relative Fold mRNA Content} = 2^{-\Delta\Delta CT}$$

Relative-fold mRNA content that was less than onefold indicating suppression of gene expression was transformed using Equation (3) to enhance the graphical representation of these data.

Equation (3):

$$\text{Relative Fold Suppression} = \frac{-1}{\text{Relative Fold mRNA Content}}$$

All mass values from hepatobiliary disposition studies were normalized to the mean protein (mg) content per well. Cellular accumulation determined in Minus (–) buffer (Cellular Accumulation_{Minus (–) Buffer}) represents the total mass of analyte inside the hepatocyte at the end of the incubation time period.¹⁷ Total accumulation determined in Plus (+) buffer (Accumulation_{Plus (+) Buffer}) represents the total mass of analyte taken up and excreted into bile pockets (Cells + Bile).¹⁷ The biliary accumulation represents the total mass of analyte excreted into the bile pockets and was calculated according to Equation (4).

Equation (4):

$$\text{Bile Accumulation} = \text{Total Accumulation}_{\text{Plus (+) Buffer}} - \text{Cellular Accumulation}_{\text{Minus (–) Buffer}}$$

The biliary excretion index (BEI) represents the fraction of the analyte taken up and excreted into the bile pockets and was calculated according to Equation (5).^{17–20}

Equation (5):

$$\text{BEI} = 100 \times \frac{\text{Bile Accumulation}_{\text{equation 4}}}{\text{Total Accumulation}_{\text{Plus (+) Buffer}}}$$

Area under the curve was calculated using Equation (6) and was utilized in the *in vitro* biliary clearance (Cl_{biliary}) determination calculated using equation 7.^{17–20} Clearance calculations were scaled to body weight using 2962 mg protein/kg body weight.²¹

Equation (6):

$$\text{AUC} = \text{Incubation Time} \times \text{Analyte Concentrations}_{\text{media}}$$

Equation (7):

$$Cl_{\text{biliary}} = \frac{\text{Bile Accumulation}_{\text{equation 4}}}{\text{AUC}_{\text{equation 6}}} (2962 \text{ mg/kg})$$

The intracellular concentration of each analyte was calculated by dividing the mass of analyte in the hepatocytes (Cellular Accumulation_{Minus (–) Buffer}) by the estimated human hepatocyte intracellular fluid volume of 7.69 $\mu\text{L}/\text{mg}$ protein as described in Equation (8).^{22,23}

Equation (8):

$$\text{ICC}_{\text{Intracellular Concentration}} = \frac{\text{Cellular Accumulation}_{\text{Minus (–) Buffer}}}{7.69 \mu\text{L}/\text{mg}}$$

Total accumulation and cellular accumulation values were generated from triplicate wells and are represented by the calculated mean and standard deviation from these biological replicates.

Results

Effect of bile acid treatment on SCHH viability

Intracellular ATP content was evaluated to assess cytotoxicity of CDCA (1, 3.16, 10, 31.6, 100, 316, and 1000 μM) and the positive control tamoxifen (10 and 50 μM). The cellular ATP content in SCHH following exposure to 10 and 50 μM tamoxifen for 72 hours was reduced to 83.4% and 0.1% of solvent control, respectively (Fig. 1). CDCA exposures from 1 to 316 μM caused a slight dose-dependent decrease in cell viability from 99.8% to 72.4%, respectively, while 1000 μM CDCA reduced viability to 1.3% of solvent control (Fig. 1).

Effect of bile acid treatment on bile acid synthesis

Using QualGro Hepatocyte Culture Induction Medium and Transporter Certified SCHH, glycine conjugates of endogenous bile acid predominated, similar to *in vivo* observations. In humans, glycine comprises ~75% of the bile acid conjugate pool resulting in a glycine:taurine ratio of ~3:1.²⁴ Total bile acid content (mass) was calculated by summing TCA, GCA, TCDCA, and GCDCA in hepatocytes (cellular accumulation), bile pockets (bile accumulation), and cell culture media. Following 72 hours of exposure to 100 μM d₅-CDCA, the total endogenous bile acid content in SCHH cultures was decreased significantly to 5.8% of control in HUM4119C, 22.1% of control in HC3-26, and 58.7% of control in HUM4122C (Fig. 2A).

FIG. 1. Cellular ATP content, a measure of cell viability, was evaluated in SCHH following exposure to increasing concentrations of solvent control and CDCA for 72 hours. Tamoxifen (10 and 50 μM) was used as a positive control. ATP content for each treatment group was determined from triplicate wells and represented by the calculated mean, while error bars represent the standard deviation from these biological replicates ($n=3$). Statistical significance was determined using Dunnett's multiple comparisons; * $p \leq 0.05$. ATP, adenosine triphosphate; SCHH, sandwich-cultured human hepatocytes.

FIG. 2. The effect of 72 hours of exposure of solvent control and 100 μ M d-5CDCA, the FXR agonist, on (A) the endogenous bile acid pool in SCHH from each of the three donors. Total bile acid content in the hepatocytes and the cell culture media was calculated, including tauro-CA, glyco-CA, tauro-CDCA, and glyco-CDCA. Data are expressed as mean percent of control of replicates ($n = 3$), while the error bars represent the standard deviation. Statistical significance was determined by Student's t-test; $*p \leq 0.001$. The effect of 72 hours of exposure of solvent control and 100 μ M CDCA on (B) mRNA content of CYP7A1, FGF19, (C) CYP8B1, and SHP in SCHH was evaluated in each of the three donors. Data are represented as mean fold change in mRNA content versus control, with error bars representing a 95% confidence interval. FGF19, fibroblast growth factor 19; FXR, farnesoid X receptor; SHP, small heterodimer partner.

Following 72 hours of exposure to 100 μ M CDCA, mRNA content of multiple genes (e.g., CYP7A1, CYP8B1, FGF19, SHP) involved with bile acid homeostasis was evaluated using TaqMan qRT-PCR. CYP7A1, the first enzyme in the biosynthesis of primary BA, mRNA content was decreased in all three SCHH preparations treated with CDCA (Fig. 2B). CYP7A1 mRNA content decreased across all three donors ranging from 158- to 1673-fold less than solvent control. Concomitant increases in the SHP and FGF19 mRNA content were also observed in SCHH treated with 100 μ M CDCA (Fig. 2B, C). Increases in SHP and FGF19 mRNA content above solvent control following treatment with 100 μ M CDCA ranged from 1.7- to 6.2-fold and 189- to 1889-fold, respectively, across all three donors. Interestingly, no marked changes were observed in CYP8B1 mRNA content in SCHH following 72 hours of exposure with 100 μ M CDCA (Fig. 2C). These data demonstrated

that treatment of SCHH with the FXR agonist, CDCA, reduced bile acid synthesis resulting in decreased bile acid content. These data were consistent with concomitant increases in SHP and FGF19 and decreases in CYP7A1 mRNA content observed in all three donors treated with 100 μ M CDCA.²⁵⁻²⁷

Effect of bile acid treatment on d₈-TCA disposition and transporter gene expression

The hepatobiliary disposition of d₈-TCA was examined following 72 hours of exposure to 100 μ M CDCA utilizing B-CLEAR technology. No significant changes in the BEI of d₈-TCA were observed with the first two SCHH preparations (HUM4119C and HC3-26), however, there was a significant increase in d₈-TCA BEI to 171% of control in SCHH cultures from HUM4122C (Fig. 3A). In contrast, the biliary clearance of d₈-TCA was significantly reduced

FIG. 3. The effect of 72 hours of exposure of solvent control and FXR agonist, 100 μ M of treated versus control CDCA, on the hepatobiliary disposition of d₈-TCA was assessed in SCHH from all three donors utilizing B-CLEAR technology. Statistical significance was determined by Student's t-test or Tukey's multiple comparisons. (A) BEI; Student's t-test $*p$ -value ≤ 0.05 , Tukey's multiple comparison $^{\$}p$ -value ≤ 0.05 , (B) biliary clearance; Student's t-test $*p$ -value ≤ 0.02 , (C) intracellular concentration; Student's t-test $*p$ -value ≤ 0.01 values were determined from triplicate wells ($n = 3$) and the results were normalized to a solvent control. Data are expressed as mean percent of control ($n = 3$), while the error bars represent the standard deviation. BEI, biliary excretion index.

across all three donors examined. The biliary clearance of d₈-TCA was reduced to 46.3% of control (1.21 to 0.56 mL/min/kg) in HUM4119C, 43.4% of control (1.38 to 0.60 mL/min/kg) in HC3-26, and 19.6% of control (0.16 to 0.03 mL/min/kg) in HUM4122C (Fig. 3B). These results were inconsistent with the increases in BSEP mRNA content observed in SCHH treated with 100 μ M CDCA for 72 hours (Fig. 4A). Increases in BSEP mRNA content in SCHH treated with 100 μ M CDCA ranged from 2.21- to 7.55-fold greater than solvent control across all three donors examined. Induction of BSEP mRNA content by CDCA was consistent with the reported FXR-dependent mechanism demonstrated to upregulate this bile acid transporter.¹³ In addition, the intracellular concentration of d₈-TCA was significantly reduced to 10.2%, 48.2%, 59.9% of solvent control across all three SCHH donors treated with 100 μ M CDCA (Fig. 3C). These results were consistent with BSEP mRNA induction; however, increases in d₈-TCA biliary efflux (BEI) were only observed in one (HUM4122C) of the three SCHH cultures following treatment with CDCA.

TaqMan qRT-PCR was used to assess the mRNA content of multiple transporters (NTCP, OATPs, MRPs, BCRP, Pgp,

BSEP, OST α , OST β) thought to be involved with bile acid transport in all three donors following 72 hours of exposure to 100 μ M CDCA. No marked changes (greater than twofold) of NTCP, OATP1B3, and OATP2B1 mRNA content were observed in any of the three SCHH preparations exposed to CDCA (Fig. 4B). Similarly, no marked changes were observed in mRNA content of the efflux transporters, including Pgp, MRP2, MRP3, MRP4, and BCRP (Fig. 4A, B). A small decrease (-2.55 -fold below control) of OATP1B1 mRNA content was observed in one of the three SCHH treated with CDCA for 72 hours (Fig. 4B). However, increases of OST α and OST β mRNA content, a basolateral heterodimer bile acid transporter, were observed across all three SCHH treated for 72 hours with 100 μ M CDCA (Fig. 4C). Increases in OST α and OST β mRNA content ranged from 3.06- to 6.76-fold and from 20.7- to 187-fold above solvent control across all three donors, respectively. These results were consistent with reports demonstrating that BSEP and OST α/β are upregulated following bile acid treatment.⁶ In addition, these results were consistent with decreases of d₈-TCA intracellular concentration in SCHH following exposure to CDCA for 72 hours. Taken together, these results suggested that bile

FIG. 4. The effect of 72 hours of exposure of solvent control and 100 μ M CDCA on (A) mRNA content of numerous bile acid biliary efflux (BCRP, BSEP, MRP2, and Pgp), (B) bile acid uptake (NTCP, OAT1B1, OAT1B3, OAT2B1, and OCT1), and (C) bile acid basolateral efflux (MRP2, MRP3, OST α , and OST β) transporters in SCHH from all three donors was assessed. Data are represented as mean-fold change in mRNA content versus control, with error bars representing a 95% confidence interval. BSEP, bile salt export pump.

acid efflux increased in SCHH exposed to 100 μ M CDCA. However, biliary clearance of d₈-TCA was reduced under these conditions, suggesting that basolateral efflux was also involved in decreasing bile acid intracellular concentration in hepatocytes following FXR activation.

Discussion

The present study utilized SCHH from three donors and B-CLEAR technology to demonstrate that basolateral efflux and not just canalicular efflux contribute to bile acid efflux from the hepatocyte following 72 hours of exposure to the FXR agonist, CDCA. The primary purpose of the study was to examine the hepatobiliary disposition changes of bile acid following FXR activation under noncytotoxic cholestatic conditions. In addition,

the present study utilized qRT-PCR to quantitatively evaluate transporter mRNA expression under these conditions to estimate individual contribution of different uptake and efflux transporters to bile acid accumulation.

CDCA has been identified as a potent endogenous FXR ligand⁴⁻⁶ and acts as an important signaling molecule to regulate bile acid content via the activation of the nuclear receptor FXR. FXR has been demonstrated to act as a master regulator that controls bile acid homeostasis through synthesis and transport.^{1,4} Identification of FXR as therapeutic target for the treatment of chronic liver diseases (e.g., primary biliary cirrhosis) has led to the development of more potent FXR agonists.²⁷ In primary biliary cirrhosis patients, total serum bile acid concentrations have been reported to range from 24 to 389 μ M.²⁸ Treatment of SCHH with increasing

concentrations of CDCA did not result in substantial loss of cell viability ($\geq 25\%$) at concentrations of $\leq 100 \mu\text{M}$, suggesting that CDCA was well-tolerated at the concentration examined. These data were supported by a qualitative morphological assessment showing no marked loss of cell to cell contact or loss of confluency in SCHH cultures (data not shown) treated with $\leq 100 \mu\text{M}$. In addition, dose-response studies in our laboratory (data not shown) and other laboratories have demonstrated that $100 \mu\text{M}$ CDCA treatment of primary hepatocytes achieved maximum response of FXR-regulated target genes involved in bile acid homeostasis.⁶

The effect of FXR activation on total endogenous bile acid content was assessed following 72 hours of exposure to the FXR agonist CDCA. Not only was the total bile acid content decreased but each individual bile acid assessed (TCA, GCA, TCDCA, and GCDCA) also decreased in all three donors upon exposure to $100 \mu\text{M}$ d₅-CDCA. These decreases in total endogenous bile acid content were concomitant with decreased CYP7A1 mRNA content and increased SHP and FGF19 mRNA content across all three donors. These results were consistent with reports demonstrating that CYP7A1 is downregulated following bile acid treatment (e.g., CDCA [6]) as a result of increased expression of SHP and FGF19 leading to the inhibition of HNF4 α transcriptional activation of the CYP7A1 gene promoter.²⁴⁻²⁶

To examine the effect of FXR activation on bile acid disposition, the hepatobiliary disposition of d₈-TCA was examined in all three donors following 72 hours of exposure to FXR agonist CDCA utilizing B-CLEAR[®] technology. Biliary clearance of d₈-TCA was expected to increase resulting from increased biliary efflux of the model bile acid due to increased BSEP expression. Surprisingly, the biliary clearance of d₈-TCA was significantly reduced in all three SCHH preparations examined, ranging from 19.6% to 46.3% of control following exposure to $100 \mu\text{M}$ CDCA. The BEI represents the fraction of a compound taken up into the hepatocyte that is excreted into the bile. Since the expression of BSEP, the transporter responsible for the biliary excretion of bile acid was increased, the BEI of d₈-TCA was also expected to increase across all donors following exposure to CDCA. However, the BEI of d₈-TCA was only increased in one of the three SCHH preparations examined. These results were inconsistent with increases in BSEP mRNA content observed in all three SCHH preparations following treatment with CDCA. Induction of BSEP mRNA content by CDCA was consistent with the reported FXR-dependent mechanism demonstrated to upregulate this canalicular bile acid efflux transporter.¹³ The expression of other canalicular efflux transporters was also examined following exposure to CDCA, including Pgp, MRP2, and BCRP; however, no marked changes (greater than twofold) of mRNA content were observed in any of the SCHH preparations studied.

Total accumulation (hepatocyte+bile) of d₈-TCA was significantly reduced in SCHH following exposure to $100 \mu\text{M}$ CDCA (data not shown). Similarly, the intracellular concentration of d₈-TCA was significantly reduced in SCHH following exposure to $100 \mu\text{M}$ CDCA. A decrease in the total accumulation suggested that uptake was decreased and was consistent with a decrease in biliary clearance and intracellular concentration of d₈-TCA. In rodent models, reduction in the expression of the uptake bile acid transporter NTCP has been demonstrated to occur under cholestatic conditions. The

downregulation of NTCP mRNA content has been demonstrated to share the same FXR/SHP-dependent regulatory pathway involved in CYP7A1 downregulation in rodents.²⁹ This mechanism would likely explain the decreases observed in the total accumulation and intracellular concentration of d₈-TCA in SCHH following exposure to FXR agonists. However, no marked changes (greater than twofold) were observed in NTCP mRNA content across all three SCHH examined under these conditions. These data suggested that a species-specific difference may exist between humans and rodents in NTCP transcriptional regulation.²⁹

To exclude other bile acid uptake transporters, the mRNA expression of OATP1B1, OATP1B3, and OATP2B1 was examined in SCHH. No changes (greater than twofold) in mRNA content of OATP1B3 and OATP2B1 were observed following treatment. A decrease of OATP1B1 mRNA content (2.55-fold below solvent control) was observed following CDCA treatment. OATPs have been reported to contribute to bile acid uptake; however, the decrease of OATP1B1 mRNA content was only observed in one of the three SCHH treated with CDCA, while decreases in d₈-TCA intracellular concentration were observed in all SCHH under the same conditions. These results suggested that the decreases observed in total accumulation and intracellular concentration of d₈-TCA were not the result of decreased expression of transporters involved in the hepatic uptake of bile acids. Alternatively, an increase in basolateral bile acid efflux would explain the observed phenomenon. Other laboratories have suggested that basolateral efflux of bile acid via MRP3 and MRP4 likely occurs under normal conditions.^{3,9,11} The contribution of basolateral transport to bile acid efflux under normal conditions was identified in a recent publication, which showed that the basolateral clearance for taurocholate was $\sim 30\%$ of the taurocholate canalicular clearance.³⁰ Basolateral efflux of bile acids is thought to increase the efficiency of bile acid biliary excretion in the liver. However, no marked increases (greater than twofold) were observed in mRNA content of MRP3 and MRP4 in any of the three SCHH preparations examined following exposure to CDCA, an FXR agonist.

Interestingly, increases in the mRNA content of the basolateral efflux heterodimer transporter, OST α/β , were observed across all three SCHH preparations treated with $100 \mu\text{M}$ CDCA. These results were consistent with reports demonstrating that hepatic OST α/β expression in patients with primary biliary cirrhosis and in bile duct-ligated rats and mice is induced via FXR-dependent mechanism.² Induction of bile acid basolateral efflux via OST α/β would be consistent with a decrease of bile acid intracellular concentrations and subsequent reduction in biliary clearance of d₈-TCA, since bile acids are endogenous substrates of OST α/β .¹² This postulation was supported by the intracellular concentration decreases of d₈-TCA in SCHH cultures exposed to $100 \mu\text{M}$ CDCA for 72 hours. Alternatively, decreases of intracellular concentration of the model bile acid, d₈-TCA, may be the result of competitive inhibition between dosed bile acids and monitored bile acid (Fig. 3C). However, d₈-TCA disposition was not performed in a coinubation with $100 \mu\text{M}$ CDCA treatment, but only after the cell culture media containing CDCA was removed and washed from the SCHH diminishing the likelihood of competitive inhibition occurring. When taken together, these data demonstrated that induction of basolateral efflux transport, OST α/β , acts as an important compensatory

pathway to decrease the hepatocyte intracellular concentration of bile acids under cholestatic conditions to prevent cholestatic hepatotoxicity.

Conclusion

The present study demonstrates that under cholestatic conditions, BSEP is likely not the principle bile acid efflux mechanism. Under cholestatic conditions, an adaptive response regulated by FXR initiates a basolateral bile acid efflux mechanism via OST α/β to reduce the intracellular concentration of bile acids in the hepatocyte (Fig. 5). These results were consistent with reports that suggest the hepatocyte adaptive response (e.g., induction of basolateral efflux transporter OST α/β) plays a protective role in cholestatic disease states (e.g., primary biliary cirrhosis).^{1,2}

As with cholestatic disease states, it is reasonable to assume that this compensatory mechanism is a potentially important pathway to prevent cholestatic drug-induced liver injury (DILI). Much focus has been placed on the development of acute interaction screening platforms to quantify BSEP inhibition potential of drug candidates in an effort to reduce the risk of cholestatic DILI in humans. However, *in vitro* BSEP inhibition potency as quantified by these *in vitro* BSEP screening platforms has not been demonstrated to correlate with *in vivo* cholestatic DILI severity.^{31,32} This compensatory mechanism may answer this paradox. Likely, inhibition of BSEP is the initial insult that triggers a transient increase in endogenous bile acids (e.g., TCDCA, GCDCA, TCA, GCA, or all), resulting in the activation of FXR and initiation of the basolateral bile acid efflux compensatory mechanism, thus preventing cholestatic

hepatotoxicity. However, inhibition/interference of this adaptive response would potentially lead to cholestatic hepatotoxicity via inhibition of basolateral bile acid efflux or antagonism of FXR activation. FXR antagonists have been identified and their potential to induce cytotoxicity was demonstrated in HepG2 cells.³³ Our results suggest that to properly assess a drug candidate's cholestatic DILI potential, an assay platform should include a fully integrated cell system (e.g., SCHH) capable of generating this adaptive response, and integrating the acute and chronic effects of the drug candidate on the hepatobiliary disposition of bile acids.

Author Disclosure Statement

No competing financial interests exist.

References

- Chiang JY. Bile acids: Regulation of synthesis. *J Lipid Res* 2009;50:1955–1966.
- Boyer JL, Trauner M, Mennone A, et al. Upregulation of a basolateral FXR-dependent bile acid efflux transporter OST α -OST β in cholestasis in humans and rodents. *Am J Physiol Gastrointest Liver Physiol* 2006;290:G1124–G1130.
- Kullak-Ublick GA, Stieger B, Meier PJ. Enterohepatic bile salt transporters in normal physiology and liver disease. *Gastroenterology* 2004;126:322–342.
- Makishima M, Okamoto AY, Repa JJ, et al. Identification of a nuclear receptor for bile acids. *Science* 1999;284:1362–1365.
- Parks DJ, Blanchard SG, Blanchard SG, et al. Bile acids: Natural ligands for an orphan nuclear receptor. *Science* 1999;284:1365–1368.
- Liu J, Lu H, Lu Y, et al. Potency of individual bile acids to regulate bile acid synthesis and transport genes in primary human hepatocyte cultures. *Toxicol Sci* 2014;141:538–546.
- Byrne JA, Strautnieks SS, Mieli-Vergani G, et al. The human bile salt export pump: Characterization of substrate specificity and identification of inhibitors. *Gastroenterology* 2002;123:1649–1658.
- Hayashi H, Takada T, Suzuki H, et al. Transport by vesicles of glycine- and taurine-conjugated bile salts and taurothiocholate 3-sulfate: A comparison of human BSEP and rat Bsep. *Biochim Biophys Acta* 2005;1738:54–62.
- Hirohashi T, Suzuki H, Takikawa H, et al. ATP-dependent transport of bile salts by rat multidrug resistance-associated protein 3 (Mrp3). *J Biol Chem* 2000;275:2905–2910.
- Akita H, Suzuki H, Ito K, et al. Characterization of bile acid transport mediated by multidrug resistance associated protein 2 and bile salt export pump. *Biochim Biophys Acta Biomembranes* 2001;1511:7–16.
- Rius M, Hummel-Eisenbeiss J, Hofmann AF, et al. Substrate specificity of human ABCC4 (MRP4)-mediated co-transport of bile acids and reduced glutathione. *Am J Physiol Gastrointest Liver Physiol* 2006;290:G640–G649.
- Ballatori N, Fang F, Christian WV, et al. Ost α -Ost β is required for bile acid and conjugated steroid disposition in the intestine, kidney, and liver. *Am J Physiol Gastrointest Liver Physiol* 2008;295:G179–G186.
- Ananthanarayanan M, Balasubramanian N, Makishima M, et al. Human bile salt export pump promoter is transactivated by the farnesoid X receptor/bile acid receptor. *J Biol Chem* 2001;276:28857–28865.
- Frankenberg T, Rao A, Chen F, et al. Regulation of the mouse organic solute transporter α - β , Ost α -Ost β , by bile

FIG. 5. (A) Schematic illustrates the disposition of bile acids (GCA, TCA, GCDCA, TCDCA) in the hepatocyte under normal conditions. Under normal conditions, bile acids are primarily excreted into the bile by BSEP, a canalicular efflux transporter. (B) Under cholestatic conditions, bile acids activate FXR leading to an increase in the expression of bile acid efflux transporters BSEP and OST α/β . Induction of OST α/β acts as a “safety-valve” to increase the efflux of bile acids protecting the hepatocyte from bile acid toxicity.

- acids. *Am J Physiol Gastrointest Liver Physiol* 2006; 290:G912–G922.
15. Guillouzo A, Morel F, Langouet S, et al. Use of hepatocyte cultures for the study of hepatotoxic compounds. *J Hepatol* 1997;26:73–80.
 16. Tyson C, Green C. Cytotoxicity measures: Choices and methods. In: *The isolated hepatocyte: use in toxicology and xenobiotic biotransformation*. E Rauchman E. G Padilla (eds); pp. 119–158. New York: Academic Press; 1987.
 17. Ghibellini G, et al. In vitro-in vivo correlation of hepatobiliary drug clearance in humans. *Clin Pharmacol Ther* 2007; 81:406–413.
 18. Chandra P, Brouwer KL. The complexities of hepatic drug transport: Current knowledge and emerging concepts. *Pharm Res* 2004;21:719–735.
 19. Liu X, et al. Correlation of biliary excretion in sandwich-cultured rat hepatocytes and in vivo in rats. *Drug Metab Dispos* 1999;27:637–644.
 20. Marion TL, et al. Endogenous bile acid disposition in rat and human sandwich-cultured hepatocytes. *Toxicol Appl Pharmacol* 2012;261:1–9.
 21. Freeman K, Brouwer KR. *QTS Technical Application Bulletin*. TAB Biol 007v2, August 8, 2012.
 22. Marion T, Brouwer KR. Determination of Hepatocellular Volume. *Qualyst Transporter Solutions Technical Application Bulletin* TAB Biol 005V2, November 29, 2011.
 23. Tagliacozzi D, Mozzi AF, Casetta B, et al. Quantitative analysis of bile acids in human plasma by liquid chromatography-electrospray tandem mass spectrometry: A simple and rapid one-step method. *Clin Chem Lab Med* 2003;41:1633–1641.
 24. Lee YK, Dell H, Dowhan DH, et al. The orphan nuclear receptor SHP inhibits hepatocyte nuclear factor 4 and retinoid X receptor transactivation: Two mechanisms for repression. *Mol Cell Biol* 2000;20:187–195.
 25. Del Castillo-Olivares A, Campos JA, Pandak WM, et al. Role of FTF/LRH-1 on bile acid biosynthesis. A known nuclear receptor activator that can act as a suppressor of bile acid biosynthesis. *J Biol Chem* 2004;279:16813–16821.
 26. Holt JA, Luo G, Billin AN, et al. Definition of a novel growth factor-dependent signal cascade for the suppression of bile acid biosynthesis. *Genes Dev* 2003;17:1581–1591.
 27. Ali AH, Carey EJ, Lindor KD. Recent advances in the development of farnesoid X receptor agonists. *Ann Transl Med* 2014;3:1–16.
 28. Murphy GM, et al. Serum bile acids in primary biliary cirrhosis. *Gut* 1972;13:201–206.
 29. Denson LA, Sturm E, Echevarria W, et al. The orphan nuclear receptor, shp, mediates bile acid-induced inhibition of the rat bile acid transporter, ntcp. *Gastroenterology* 2001; 121:140–147.
 30. Yang K, Pfeifer ND, Kock K, et al. Species differences in hepatobiliary disposition of taurocholic acid in human and rat sandwich-cultured hepatocytes: Implications for drug-induced liver injury. *J Pharmacol Exp Ther* 2015;353: 415–423.
 31. Morgan RE, Trauner M, Staden CJ, et al. Interference with bile salt export pump function is a susceptibility factor for human liver injury in drug development. *Toxicol Sci* 2010;118:485–500.
 32. Dawson S, Stahl S, Paul N, et al. *In vitro* inhibition of the bile salt export pump correlates with risk of cholestatic drug-induced liver injury in humans. *Drug Metab Dispos* 2012;40:130–138.
 33. Lu W, Cheng F, Jiang J, et al. FXR antagonism of NSAIDs contributes to drug-induced liver injury identified by systems pharmacology approach. *Sci Rep* 2015;5:8114.

Address correspondence to:
 Dr. Jonathan P. Jackson
 Qualyst Transporter Solutions
 2810 Meridian Parkway
 Suite 100
 Durham, NC 27713

E-mail: jonathanjackson@qualyst.com